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Dependence of Pituitary Hormone Secretion on the Pattern of Spontaneus Voltage-gated Calcium Influx

CELL TYPE-SPECIFIC ACTION POTENTIAL SECRETION COUPLING*

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In excitable cells, voltage-gated calcium influx provides an effective mechanism for the activation of exocytosis. In this study, we demonstrate that although rat anterior pituitary lactotrophs, somatotrophs, and gonadotrophs exhibited spontaneous and extracellular calcium-dependent electrical activity, voltage-gated calcium influx triggered secretion only in lactotrophs and somatotrophs. The lack of action potential-driven secretion in gonadotrophs was not due to the proportion of spontaneously firing cells or spike frequency. Gonadotrophs exhibited calcium signals during prolonged depolarization comparable with signals observed in somatotrophs and lactotrophs. The secretory vesicles in all three cell types also had a similar sensitivity to voltage-gated calcium influx. However, the pattern of action potential calcium influx differed among three cell types. Spontaneous activity in gonadotrophs was characterized by high amplitude, sharp spikes that had a limited capacity to promote calcium influx, whereas lactotrophs and somatotrophs fired plateau-bursting action potentials that generated high amplitude calcium signals. Furthermore, a shift in the pattern of firing from sharp spikes to plateau-like spikes in gonadotrophs triggered luteinizing hormone secretion. These results indicate that the cell type-specific action potential secretion coupling in pituitary cells is determined by the capacity of their plasma membrane oscillator to generate threshold calcium signals.

Although anterior pituitary secretory cells are derived from the same progenitor cells, they differ with respect to their secretory patterns *in vitro* and *in vivo. In vitro*, basal prolactin (PRL)[1] and growth hormone (GH) secretion from pituitary fragments, dispersed pituitary cells, and immortalized lacto-somatotrophs is high and is dependent on the extracellular calcium concentration (1–4). In contrast, basal luteinizing hormone (LH) secretion is low and not dependent on the extracellular calcium concentration (1). *In vivo*, animals bearing ectopic pituitary grafts release high levels of PRL and low levels of LH for a prolonged period, leading to pseudo-pregnancy (5). Because of the high levels of basal GH and PRL secretion, it is not surprising that lactotrophs and somatotrophs are under negative hypothalamic control by $G_{i/o}$-coupled dopamine and somatostatin receptors, in addition to positive control by Ca^{2+}-mobilizing and G_s-coupled receptors, such as GH-releasing hormone and thyrotropin-releasing hormone receptors. On the other hand, LH secretion from gonadotrophs is under positive hypothalamic control by Ca^{2+}-mobilizing receptors, including gonadotropin-releasing hormone (GnRH) and endothelin-A, but no inhibitory hypothalamic factor has been identified (6, 7).

It is not known what endows lactotrophs and somatotrophs, but not gonadotrophs, with the ability to secrete high levels of hormone in the absence of any stimuli. One possibility is that lactotrophs and somatotrophs fire spontaneous action potentials (APs) that are capable of driving sufficient Ca^{2+} entry to stimulate hormone secretion, whereas gonadotrophs are quiescent in the absence of any stimuli. Consistent with this, cultured somatotrophs (8, 9), lactotrophs (10, 11), and immortalized GH cells (12–16), as well as *in situ* somatotrophs (17), spontaneously fire APs, and the majority of unstimulated male rat gonadotrophs are quiescent (18). In ovariectomized rats, however, gonadotropin secretion remained low despite the observation that about 50% of the cells examined exhibited spontaneous AP firing (1, 19). These observations raise the possibility that the nature of spontaneous AP firing, such as Ca^{2+}-dependent *versus* Na^+-dependent spiking, or variations in the proportion of excitable cells, and/or frequency of spontaneous firing account for the cell type-specific patterns of basal hormone secretion. Finally, the differences in the patterns of basal hormone secretion may be due to differences in the ability of voltage-gated Ca^{2+} influx (VGCI) to increase intracellular calcium concentration ($[Ca^{2+}]_i$) and stimulate secretion in spontaneously active cells. In male gonadotrophs, for example, short membrane depolarization and the ensuing increase in $[Ca^{2+}]_i$ do not stimulate exocytosis (20), whereas a prolonged membrane depolarization by high potassium is sufficient to stimulate secretion in several anterior pituitary cell types, including gonadotrophs (1, 21). Thus, the profile of the AP wave form, *i.e.* the AP duration, may determine the amplitude of the $[Ca^{2+}]_i$ and secretory responses.

In the present study, we examined the patterns of AP-driven Ca^{2+} entry and their relationship to basal hormone secretion in each cell type under identical culture and recording conditions. Spontaneous electrical membrane activity and $[Ca^{2+}]_i$ were recorded simultaneously to determine the ability of AP firing in each cell type to drive VGCI. To monitor basal hormone secre-

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[1] The abbreviations used are: PRL, prolactin; AP, action potential; VGCC, voltage-gated calcium channel; VGCI, voltage-gated calcium influx; GH, growth hormone; LH, luteinizing hormone; GnRH, gonadotropin-releasing hormone; TTX, tetrodotoxin.

tion and its dependence on AP-driven Ca^{2+} entry at a similar time scale to that used in electrophysiological experiments, a rapid perifusion system was used. Our results indicate specific profiles of the AP wave forms in three cell types, and their ability to drive Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCCs) accounts for the cell type-specific patterns of basal hormone secretion. Specifically, gonadotrophs fired sharp, high amplitude APs with a limited capacity to drive Ca^{2+} influx, whereas lactotrophs and somatotrophs exhibited plateau-bursting activity that had a high capacity to drive Ca^{2+} entry.

EXPERIMENTAL PROCEDURES

Cell Cultures and Treatments—Experiments were performed on anterior pituitary cells from normal postpuberal female Harlan Sprague-Dawley rats obtained from Taconic Farm (Germantown, NY). Pituitary cells were dispersed as described previously (22) and cultured as mixed cells or enriched lactotrophs, somatotrophs, and gonadotrophs in medium 199 containing Earle's salts, sodium bicarbonate, 10% heat-inactivated horse serum, and antibiotics. A two-stage Percoll discontinuous density gradient procedure (22) was used to obtain enriched lactotroph and somatotroph populations. Somatotrophs were further identified by their cell type-specific morphologies and by their responses to GH-releasing hormone. In enriched lactotroph populations, lactotrophs were further identified by their cell type-specific morphology and by the addition of thyrotopin-releasing hormone. Gonadotrophs were initially identified by their cell type-specific morphology and, subsequent to experimentation by addition of GnRH, which stimulates small conductance, Ca^{2+}-activated K^+ current and $[Ca^{2+}]_i$ oscillations only in gonadotrophs (23, 24).

Hormone secretion was monitored using rapid cell column perifusion experiments as previously described (25). Briefly, 1.5×10^7 cells were incubated with preswollen cytodex-1 beads in 60-mm Petri dishes for 2 days. The beads were then transferred to 0.5-ml chambers and perifused with Hanks' M199 containing 20 mM HEPES and 0.1% bovine serum albumin for 2 h at a flow rate of 0.8 ml/min at 37 °C to establish a stable basal secretion. During the experiment, 1-min fractions were collected, stored at −20 °C, and later assayed for GH, PRL, and LH content using radioimmunoassay. All reagents and standards were provided by the National Pituitary Agency and Dr. Parlow. Standard curves for three radioimmunoassays were constructed in a concentration range of 1–100 nM, and the displacement of labeled hormones with unlabeled hormones was done at 30% specific binding. The averaged IC_{50} was 6.30 ± 0.44 (n = 9), 6.52 ± 0.48 (n = 9), and 6.93 ± 0.73 (n = 8) ng/ml for GH, PRL, and LH, respectively, indicating similar sensitivity of three radioimmunoassays. To account for differences in the total number of the three hormone secreting cell types found in the anterior pituitary, hormone content from the same samples was measured and then normalized to the percentage of each cell type occurring in mixed cell populations.

Immunocytochemistry of Rat Anterior Pituitary Cells—To normalize hormone secretion to the total number of each cell type in the anterior pituitary, immunostaining of GH, LH, and PRL was performed using a avidin-biotin (ABC) peroxidase method. Dispersed cells were plated at a density of 200,000 cells/slide, fixed in Bouin's fluid for 20 min, thoroughly washed, dehydrated, and kept dry at −70 °C. On the day of immunocytochemical processing, fixed cells were sequentially rehydrated, treated with 3% H_2O_2, rinsed in phosphate-buffered saline, blocked by incubation in 10% normal goat serum in phosphate-buffered saline, washed, and incubated overnight at 4 °C with rabbit anti-LH serum (1:75,000; NIDDK, National Institutes of Health), monkey anti-GH serum (1:75,000; NIDDK, National Institutes of Health), or rabbit anti-PRL serum (1:75,000; National Pituitary Agency). On the second day, the slides were rinsed and then incubated for 1 h at room temperature with goat anti-rabbit IgG-biotin conjugate (1:9,000 dilution; Vector Laboratories Inc., Burlingame, CA), or goat anti-human IgG-biotin conjugate (1:10,000 dilution; Vector Laboratories). This was followed by avidin-biotin-peroxidase complex incubation for 45-min period at room temperature. Specific staining was visualized with a diaminobenzidine substrate kit for peroxidase (Vector Laboratories). Antibody specificity was determined by incubating cells with LH, GH, or PRL antiserum preabsorbed with homologous or related peptides.

Electrophysiological Measurements—Current and voltage clamp recordings were performed at room temperature using an Axopatch 200 B patch clamp amplifier (Axon Instruments, Foster City, CA) and were low pass filtered at 2 kHz. Membrane potential (V_m) was measured using the perforated patch recording technique (26). Briefly, an amphotericin B (Sigma) stock solution (60 mg/ml) was prepared in Me_2SO and stored for up to 1 week at −20 °C. Just prior to use, the stock solution was diluted in pipette solution and sonicated for 30 s to yield a final amphotericin B concentration of 240 μg/ml. Patch electrodes used for perforated patch recordings were fabricated from borosilicate glass (outer diameter, 1.5 mm; World Precision Instruments, Sarasota, FL) using a Flaming Brown horizontal puller (P-87; Sutter Instruments, Novato, CA). Electrodes were heat polished to a final tip resistance of 3–6 MΩ and then coated with Sylgard (Dow Corning Corporation, Midland, MI) to reduce pipette capacitance. Pipette tips were briefly immersed in amphotericin B-free solution and then backfilled with the amphotericin B-containing solution. A series resistance of <15 MΩ was reached 10 min following the formation of a gigaohm seal (seal resistance > 5 GΩ) and remained stable for up to 1 h. Pulse generation, data acquisition and analysis were done with a PC equipped with a Digidata 1200 A/D interface in conjunction with Clampex 8 (Axon Instruments). For recording V_m, the extracellular medium contained 120 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 4.7 mM KCl, 0.7 mM $MgSO_4$, 10 mM glucose, and 10 mM HEPES (pH adjusted to 7.4 with NaOH), and the pipette solution contained 50 mM KCl, 90 mM K^+-aspartate, 1 mM $MgCl_2$, and 10 mM HEPES (pH adjusted to 7.2 with KOH). The bath contained <500 μl of saline and was continuously perifused at a rate of 2 ml/min using a gravity-driven perfusion system.

Simultaneous Recording of $[Ca^{2+}]_i$ and V_m—Pituitary cells were incubated for 15 min at 37 °C in phenol red-free medium 199 containing Hanks' salts, 20 mM sodium bicarbonate, 20 mM HEPES, and 0.5 μM indo-1 acetoxymethyl ester (Molecular Probes, Eugene, OR). The V_m was recorded as described above, and bulk $[Ca^{2+}]_i$ was simultaneously monitored using a Nikon photon counter system as previously described (27). The Vm and bulk $[Ca^{2+}]_i$ were captured simultaneously at rate of 5 kHz using a PC equipped with a Digidata 1200 A/D interface in conjunction with Clampex 8 (Axon Instruments). The $[Ca^{2+}]_i$ was calibrated *in vivo* according to Kao (28), and the values for R_{min}, R_{max}, $S_{f,480}/S_{b,480}$ and K_d were determined to be 0.75, 3.40, 2.45, and 230 nM, respectively.

RESULTS

Extracellular Ca^{2+} Dependence of Basal Hormone Release—The pattern of basal GH, PRL, and LH secretion from dispersed anterior pituitary cells was compared using rapid perifusion (1-min fractions) experiments. Basal hormone secretion was normalized to account for differences in the size of somatotroph, lactotroph, and gonadotroph populations in mixed anterior pituitary cell preparations (Fig. 1*A*; see "Experimental Procedures" for details). In all of the experiments, the level of GH and PRL release was severalfold higher than that of LH release, *i.e.* 50–70 ng/ml for GH and PRL and below 1 ng/ml for LH. The normalized secretory profiles for each hormone from a representative experiment and the mean ± S.E. from 10 separate experiments are shown in Fig. 1*B*. These results demonstrate that basal GH and PRL secretion from perifused anterior pituitary cells is ≈ 25- and 40-fold higher, respectively, than LH secretion.

To investigate the involvement of voltage-gated Na^+ and Ca^{2+} channels in controlling basal GH, PRL, and LH secretion, we used blockers of these channels. Application of the specific voltage-gated Na^+ channel blocker, TTX (1 μM), did not alter the pattern of basal GH, PRL, or LH secretion (Fig. 2*A*), indicating that these channels are not involved in the regulation of basal pituitary hormone secretion. In contrast, application of the L-type calcium channel blocker, nifedipine, and the nonspecific Ca^{2+} channel blocker, Cd^{2+}, inhibited basal GH and PRL secretion but did not alter basal LH secretion (Fig. 2, *B* and *C*). Similarly, extracellular Ca^{2+} removal abolished GH and PRL secretion without affecting the pattern of basal LH secretion (Fig. 2*D*). These results indicate that the main fraction of basal GH and PRL secretion from perifused anterior pituitary cells is due to regulated, Ca^{2+}-dependent exocytosis in response to VGCI. The residual, Ca^{2+}-independent GH and PRL secretion, as well as total basal LH secretion, could be due to constitutive exocytosis or nonspecific leak of hormones dur-

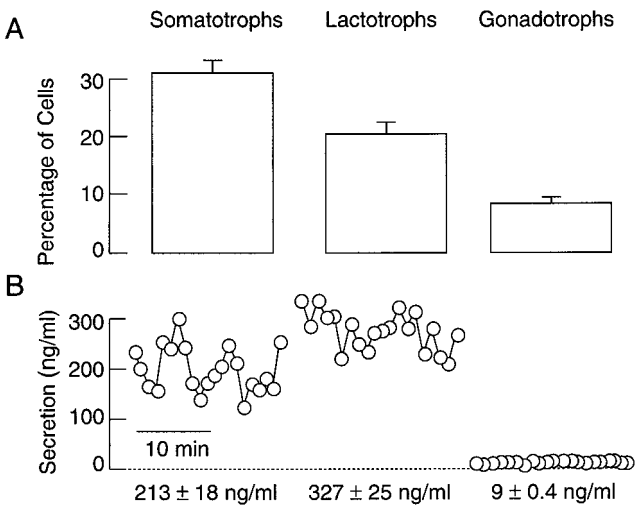

FIG. 1. **Characterization of basal GH, PRL, and LH release in perifused pituitary cells from postpuberal female rats.** *A*, percentage of immunoreactive GH-, PRL-, and LH-positive cells in mixed cultures. The values are the means ± S.E. from four experiments. *B*, basal hormone secretion in perifused cells. The *graphs* illustrate typical patterns of secretion, and the *numbers* below the *graphs* are the means ± S.E. from 10 independent experiments. In this and the following figures, secretion was analyzed in cells perifused at flow rate of 0.8 ml/min, and basal secretion was normalized to account for a difference in the size of somatotroph, lactotroph, and gonadotroph populations (see "Experimental Procedures").

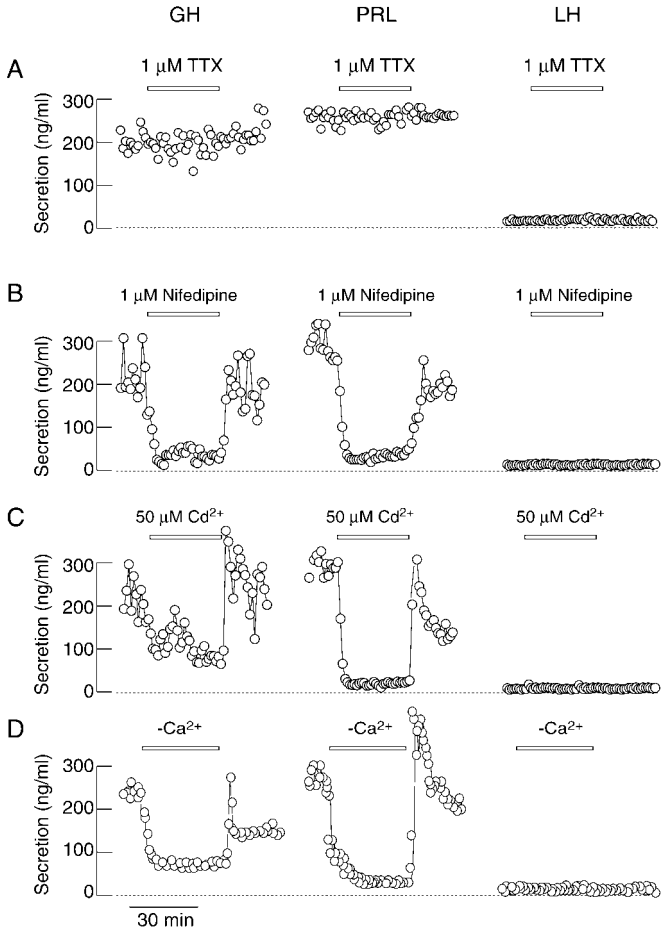

FIG. 2. **Extracellular calcium dependence of basal hormone release in perifused pituitary cells.** *A*, the lack of effects of TTX, a specific blocker of voltage-gated Na^+ channels, on basal hormone secretion. *B*, inhibition of basal hormone secretion by nifedipine, an L-type Ca^{2+} channel blocker. *C*, effects of Cd^{2+}, a nonselective VGCC-blocker, on basal secretion. *D*, effects of removal of extracellular Ca^{2+} on basal hormone secretion. The cells were perifused with Ca^{2+}-deficient medium containing 100 μM EGTA.

ing perifusion. In further experiments, we focused on VGCI-dependent basal hormone secretion.

Excitability of Pituitary Cells and Basal Secretion—The involvement of VGCCs in regulating GH and PRL secretion, but not LH secretion, could be due to the inability of gonadotrophs to fire spontaneous APs. To test this, we compared the electrical membrane activity in all three hormone-secreting cell types under identical recording conditions using the perforated patch whole cell configuration. Spontaneous AP firing with a frequency of ≈0.3 Hz was observed in a majority (>80%) of the somatotrophs and lactotrophs examined (Fig. 3*A*). In contrast, half of the gonadotrophs examined exhibited spontaneous AP firing with a frequency of 0.7 Hz (Fig. 3*A*). To test whether the lower percentage of gonadotrophs exhibiting spontaneous electrical activity accounts for the low levels of basal LH secretion compared with that of GH and PRL, we increased the percentage of gonadotrophs firing APs by the addition of 5 mM K^+ to 4.7 mM K^+-containing M199. Potassium-induced membrane depolarization increased spike frequency in spontaneously active gonadotrophs (Fig. 3*B*, *left traces*) but did not alter the profile of the AP wave form (Fig. 3*C*, *left traces*). In addition, K^+-induced membrane depolarization initiated firing in all quiescent gonadotrophs examined (Fig. 3*B*, *right traces*). These changes in V_m were accompanied with a small (less than 100 nM) increase in $[Ca^{2+}]_i$ (Fig. 3*B*, *bottom traces*). Despite the changes in the pattern of AP firing, application of 5 mM K^+ did not trigger LH secretion, whereas it increased GH and PRL secretion in the same fractions. Moreover, the level of LH secretion remained lower than that of both GH and PRL secretion (Fig. 3*C*, *right panel*).

In further experiments, we examined whether differences in the ionic mechanisms of AP firing (Ca^{2+}-dependent *versus* Na^+-dependent spiking), the profile of the AP wave form, and/or the capacity of AP firing to drive extracellular Ca^{2+} entry accounts for the cell type-specific patterns of hormone secretion. To do this, we simultaneously monitored V_m activity and $[Ca^{2+}]_i$ in all three cell types under identical recording conditions. In spontaneously active somatotrophs and lactotrophs, extracellular Ca^{2+} removal abolished AP firing and markedly decreased $[Ca^{2+}]_i$ (Fig. 4, *left* and *center traces*). In spontaneously active gonadotrophs, extracellular Ca^{2+} removal also abolished AP firing but had only a minor effect on the already low levels of basal $[Ca^{2+}]_i$ (Fig. 4, *right traces*). Thus, although all three cell types fired Ca^{2+}-dependent APs, their capacity to drive extracellular Ca^{2+} entry is greater in somatotrophs and lactotrophs than in gonadotrophs.

We next examined whether differences in the profile of the AP wave form account for the cell type-specific AP-driven Ca^{2+} signals. Somatotrophs and lactotrophs fired low amplitude, plateau-bursting APs with a duration of ≈1.3 and 0.75 s, respectively (Fig. 5, *left* and *center traces*). In contrast, gonadotrophs fired high amplitude, single spikes (Fig. 5, *right traces*) with a duration at one-half the amplitude of < 50 ms. The two patterns of AP firing, plateau-bursting *versus* single spiking, had different capacities to drive extracellular Ca^{2+} influx via VGCCs. The spontaneous plateau-bursting in somatotrophs and lactotrophs generated high amplitude $[Ca^{2+}]_i$ signals that ranged from 0.3 to 1.2 μM, whereas spontaneous single spiking in gonadotrophs generated low amplitude $[Ca^{2+}]_i$ signals ranging from 20 to 70 nM (Fig. 5).

To test whether the AP duration alone accounts for their different capacities to drive Ca^{2+} influx, somatotrophs, lactotrophs, and gonadotrophs were depolarized to −10 mV for variable times (from 25 ms to 2 s), and the accompanying

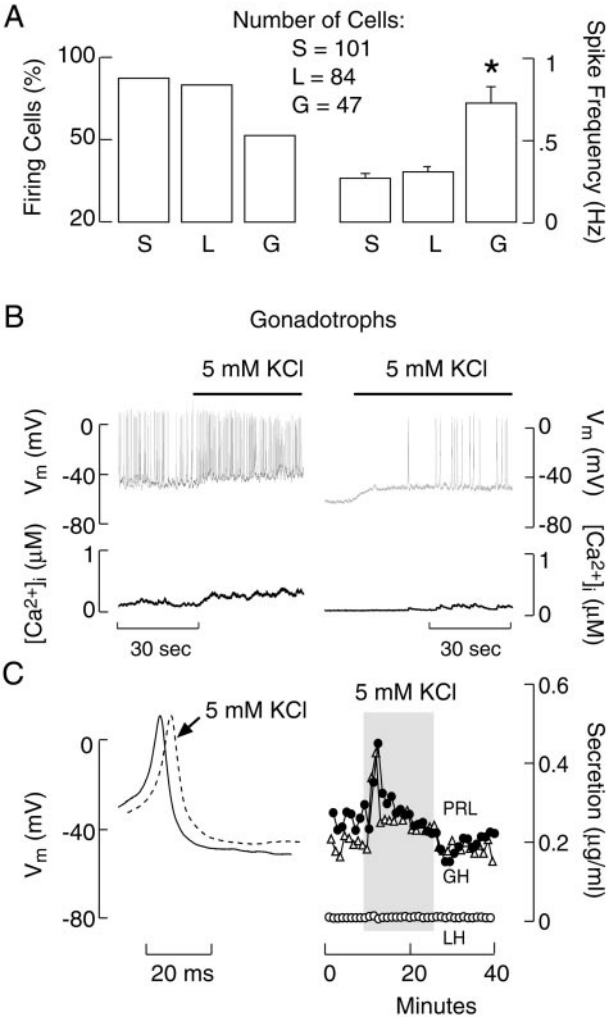

FIG. 3. **Spontaneous and potassium-induced firing of APs and secretion in pituitary cells.** *A*, *left panel*, percentage of spontaneously firing cells. *Right panel*, the averaged spike frequency, measured during 2-min period. *S*, somatotrophs; *L*, lactotrophs; *G*, gonadotrophs. *, $p < 0.01$ *versus* S and L. *B*, effects of addition of 5 mM K^+ to 4.7 mM K^+-containing medium on V_m and $[Ca^{2+}]_i$ in spontaneously active (*left traces*) and quiescent (*right traces*) gonadotrophs. *C*, *left panel*, pattern of APs in spontaneously active gonadotrophs before (*solid line*) and during (*dashed line*) K^+ depolarization. *Right panel*, effects of 5 mM K^+ on basal hormone release.

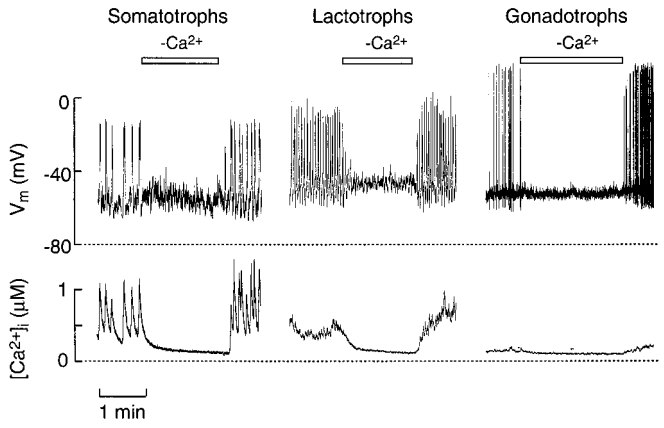

FIG. 4. **Extracellular Ca^{2+} sensitivity of spontaneous electrical activity in pituitary cells.** Simultaneous measurements of V_m and $[Ca^{2+}]_i$ in single somatotrophs, lactotrophs, and gonadotrophs. The cells were perfused with Ca^{2+}-containing (1.8 mM) and Ca^{2+}-deficient (100 nM) medium. The experiments were done with purified somatotrophs, lactotrophs, and gonadotrophs from the same preparation (see "Experimental Procedures"). At the end of the experiments, the cells were stimulated with GH-releasing hormone, thyrotropin-releasing hormone, and GnRH, the specific agonists for somatotrophs, lactotrophs/thyrotrophs, and gonadotrophs, respectively.

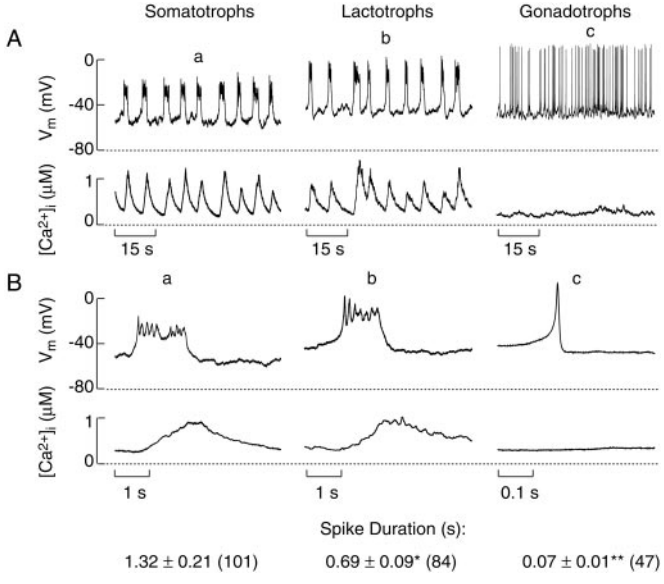

FIG. 5. **Characterization of spontaneous firing of APs in pituitary cells.** *A* and *B*, simultaneous measurements of V_m and $[Ca^{2+}]_i$ in somatotrophs, lactotrophs, and gonadotrophs. *Panels a–c* in *A* indicate APs that are shown on expanded time scale in *B*. Notice the difference in time scale for the gonadotrophs in *B*. The *numbers* below the *tracings* indicate the average duration of AP spikes, measured at half amplitude. *, $p < 0.01$ *versus* somatotrophs; **, $p < 0.01$ *versus* lactotrophs.

increase in $[Ca^{2+}]_i$ was monitored (Fig. 6*A*). In all three hormone-secreting cell types, the peak amplitude in the $[Ca^{2+}]_i$ increased progressively with an increase in the duration of the depolarizing membrane potential step. A similar increase in the peak $[Ca^{2+}]_i$ was observed between somatotrophs and gonadotrophs, whereas a lower $[Ca^{2+}]_i$ response was observed in lactotrophs (Fig. 6, *B–D*). Nevertheless, these results indicate that the duration of VGCI alone accounts for the cell type-specific patterns of AP-driven Ca^{2+} signaling.

Dependence of Basal Hormone Release on the Pattern of Firing—Our results indicate that the prolonged duration of the AP wave form in somatotrophs and lactotrophs account for the high amplitude $[Ca^{2+}]_i$ signals and the high levels of basal hormone secretion. To test whether an increase in the duration of the AP wave form in spontaneously active gonadotrophs can increase AP-driven Ca^{2+} entry and stimulate LH secretion, we used the L-type Ca^{2+} channel agonist, Bay K 8644. In spontaneously active somatotrophs and lactotrophs, the addition of 1 μM Bay K 8644 increased the frequency of firing (Fig. 7*A*, *upper traces*) and the base-line $[Ca^{2+}]_i$ (Fig. 7, *A* and *B*, *bottom traces*). In addition, Bay K 8644 application increased GH and PRL secretion (Fig. 7*C*). In spontaneously active gonadotrophs, Bay K 8644 increased the frequency of spiking and the duration of the AP wave form (Fig. 7*B*, *e versus f*). These changes in the pattern of AP firing elevated $[Ca^{2+}]_i$ to the levels observed in unstimulated somatotrophs and lactotrophs (Fig. 7*A*, *dashed line*). Moreover, the Bay K 8644-induced increase in AP-driven Ca^{2+} entry was sufficient to trigger calcium-dependent LH secretion. As shown in Fig. 7*C*, Bay K 8644-induced LH secretion was comparable with that observed in untreated somatotrophs and lactotrophs (*dashed line*). These results indicate that basal pituitary hormone secretion is dependent on the duration of the AP wave form, which determines their capacity to drive Ca^{2+} entry through VGCCs.

Steady-state Depolarization and Secretion—We next compared the capacity of VGCI to stimulate hormone secretion in

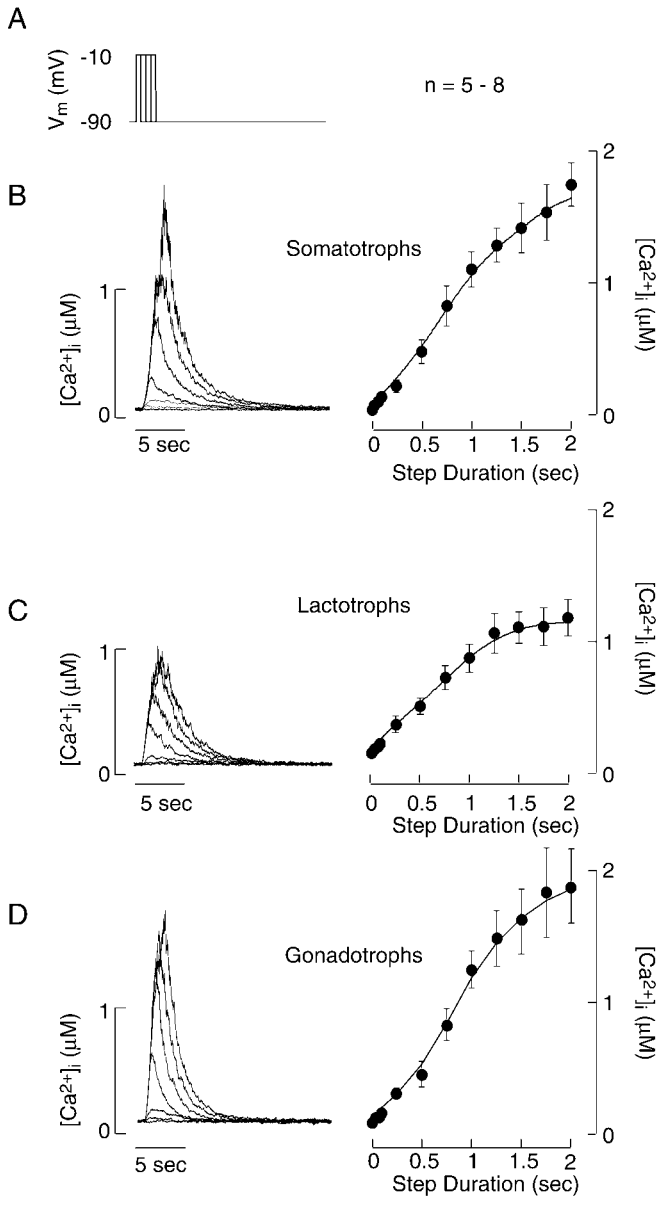

FIG. 6. **Depolarization-induced rise in $[Ca^{2+}]_i$ in pituitary cells.** *A*, cells were clamped at −90 mV and transiently (25 ms to 2 s) depolarized to −10 mV. *B–D*, *left panels*, typical profiles of $[Ca^{2+}]_i$ responses to depolarizing pulses of variable duration. *Right panels*, the relationship between peak $[Ca^{2+}]_i$ responses and duration of depolarizing pulses (means ± S.E. values). Depolarization of cells for 0.75–2 s induced a significantly lower amplitude of $[Ca^{2+}]_i$ responses in lactotrophs, compared with somatotrophs and gonadotrophs ($p < 0.05$).

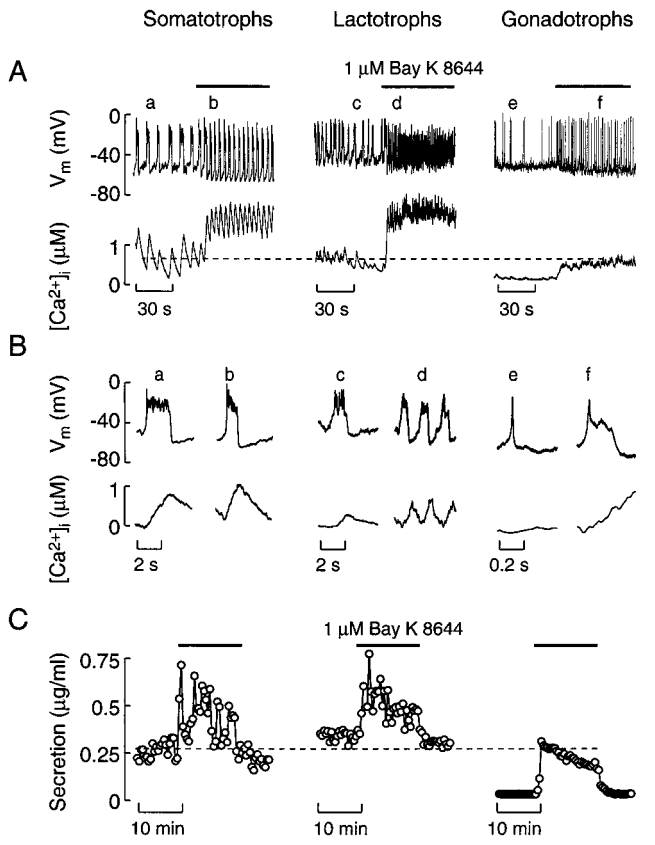

FIG. 7. **Effects of Bay K 8644, an L-type Ca^{2+} channel agonist, on V_m, $[Ca^{2+}]_i$, and hormone secretion in pituitary cells.** *A*, effects of Bay K 8644 on V_m and $[Ca^{2+}]_i$ in identified somatotrophs, lactotrophs, and gonadotrophs. The *dashed line* indicates basal $[Ca^{2+}]_i$ in somatotrophs and lactotrophs. *B*, expanded time scales, showing selected APs, labeled as *a–f*. *C*, effects of Bay K 8644 on basal hormone secretion. The *dashed line* indicates basal GH secretion.

each hormone-secreting cell type. To do this, we examined whether the levels of $[Ca^{2+}]_i$ and hormone secretion in response to steady-state V_m depolarization were similar between the three cell types. In gonadotrophs, K^+-induced V_m depolarization stimulated a dose dependent increase in LH secretion (Fig. 8*A*). Similar results were observed in somatotrophs and lactotrophs (data not shown). Sustained membrane depolarization in all three cell types by addition of 50 mM K^+ evoked a similar rise in $[Ca^{2+}]_i$ (Fig. 8*B*). Moreover, the normalized secretory response to 50 mM K^+ was comparable in all three hormone-secreting cell types (Fig. 8*C*). These results argue against the hypothesis that secretory vesicles in gonadotrophs are less sensitive to VGCI compared with that in somatotrophs and lactotrophs. They also suggest that secretory vesicles in lactotrophs are more sensitive to VGCI compared with somatotrophs, because the $[Ca^{2+}]_i$ response to V_m depolarization in lactotrophs was consistently smaller than that in somatotrophs and lactotrophs (Figs. 6*C* and 8*B*), whereas basal (Figs. 1 and 2) and K^+-induced (Fig. 8*C*) PRL secretion was higher.

DISCUSSION

In this study, we examined the ionic mechanisms underlying the different patterns of basal hormone secretion from anterior pituitary somatotrophs, lactotrophs, and gonadotrophs. In general, unstimulated cells secrete in a constitutive and regulated manner, the latter through AP-driven Ca^{2+} influx and Ca^{2+}-dependent exocytosis (29–32). Our results using perifused anterior pituitary cells indicated that basal GH and PRL secretion was much higher than basal LH secretion. As in other studies (3, 4, 9), the majority of basal GH and PRL secretion was extracellular Ca^{2+}-dependent and sensitive to blockade of VGCI through L-type channels. On the other hand, extracellular Ca^{2+} removal or VGCC blocker did not alter basal LH release. Basal hormone secretion from all three cell types was unaffected by the voltage-gated Na^+ channel blocker, TTX. These results indicate that Ca^{2+} influx via VGCCs accounts for basal GH and PRL release but not basal LH secretion.

Consistent with VGCI-dependent GH and PRL secretion shown here and by others (2, 8–11), somatotrophs and lactotrophs fire APs spontaneously. The TTX insensitivity and Ca^{2+} sensitivity of spontaneous firing of APs in somatotrophs and lactotrophs and basal GH and PRL secretion further indicates that VGGCs are major players in AP-dependent hormone secretion. However, our data show that gonadotrophs from normal females also exhibit spontaneous and extracellular Ca^{2+}-dependent excitability but not AP-dependent secretion. These

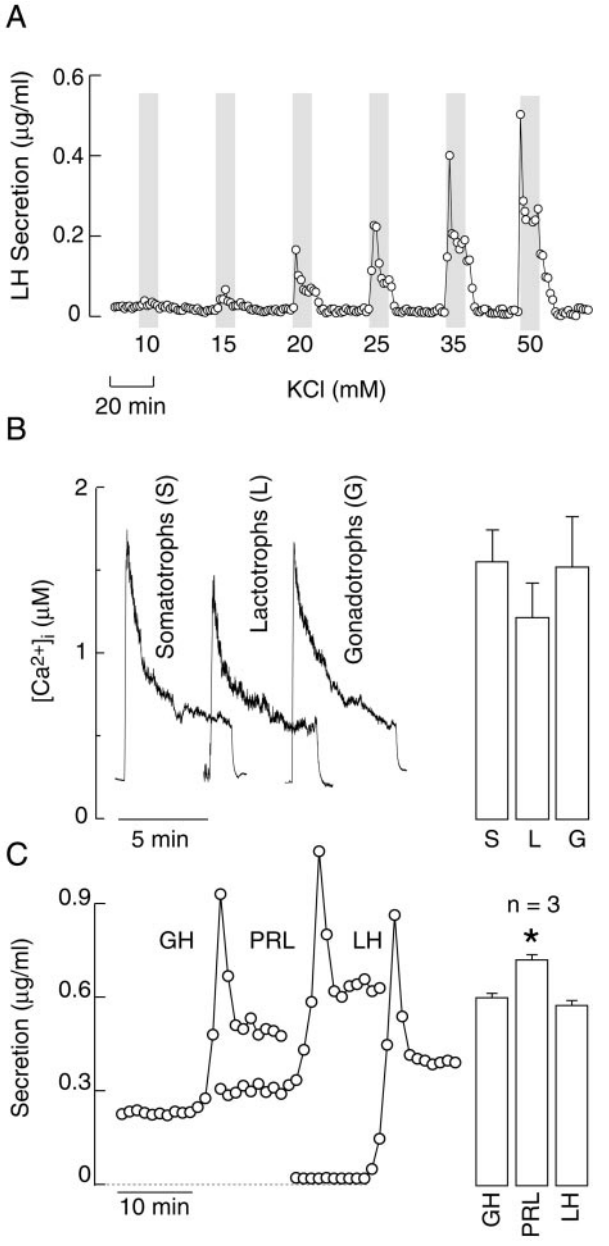

FIG. 8. **Steady-state depolarization and basal hormone secretion in pituitary cells.** *A*, dose-dependent effects of K^+ on LH release. The *gray areas* indicate the time of exposure to elevated K^+. *B*, typical traces of 50 mM K^+-induced $[Ca^{2+}]_i$ responses in somatotrophs, lactotrophs, and gonadotrophs. The *bars* indicate the means ± S.E. of peak $[Ca^{2+}]_i$ responses *C*, profiles of GH, PRL, and LH secretion, measured from the same samples. The *bars* indicate the mean values ± S.E. of hormone secretion during the first 10 min of depolarization. In all panels, the K^+ concentrations indicated were added to medium 199 already containing 4.7 mM K^+. *S*, somatotrophs; *L*, lactotrophs; *G*, gonadotrophs.

data argue against the hypothesis that the lack of spontaneous excitability of gonadotrophs accounts for low basal LH release. Also, basal LH secretion was not related to the number of cells exhibiting spontaneous firing of APs nor to the frequency of spontaneous firing, because depolarization of cells with 5 mM K^+ initiated firing in quiescent gonadotrophs and increased frequency of firing in spontaneously active cells but did not initiate LH release. Thus, although spontaneous AP firing was observed in all three-cell types, only GH and PRL were dependent on AP-driven Ca^{2+} entry.

The ability of AP to trigger secretion depends, in part, on the distance between secretory vesicles and VGCCs. In synapses, predocked release-ready vesicles are molecularly linked to calcium channels (33, 34), which facilitates their rapid release in response to VGCI (35). In contrast, single APs trigger only a minor amount of secretion in chromaffin cells, whereas a prolonged step depolarization induces massive secretion that persists after VGCI has stopped (36). In rat melanotrophs, the distance between secretory vesicles and VGCCs is also large. As a result, short (40 ms) depolarizations evoked only a minor amount of secretion (37). Single cell secretory studies, using capacitance measurements, in male rat gonadotrophs also indicate that short V_m depolarizations are insufficient to stimulate exocytosis (20).

These experiments raised the possibility that secretory vesicles in somatotrophs, lactotrophs, and gonadotrophs differ in their sensitivity to VGCI, *i.e.* that secretory vesicles in somatotrophs and lactotrophs are close to VGCCs, whereas in gonadotrophs the localized VGCI cannot reach them. However, the results shown here indicate the opposite. The $[Ca^{2+}]_i$ response to square depolarizing pulses in duration of 50 ms to 2 s were comparable in the three cell types. This is in accord with earlier published results indicating that L-type Ca^{2+} channel density is similar among the three cell types (27). Furthermore, gonadotrophs, lactotrophs, and somatotrophs exhibited comparative $[Ca^{2+}]_i$ and secretory responses during steady-state depolarization of cells with 50 mM K^+, indicating that the secretory vesicles in gonadotrophs, as in somatotrophs and lactotrophs, respond to high amplitude VGCI signals. Therefore, like chromaffin cells and melanotrophs, all three anterior pituitary cell types require global $[Ca^{2+}]_i$ signaling to trigger substantial exocytosis.

Activation of exocytosis in unstimulated cells appears to be determined by the duration of AP wave form and its capacity to drive global Ca^{2+} signals. Somatotrophs and lactotrophs exhibit plateau-bursting activity, which leads to prolong activation of L-type channels, and sustain Ca^{2+} influx and hormone secretion. On the other hand, gonadotrophs fire single APs with a limited capacity to elevate $[Ca^{2+}]_i$ and stimulate hormone secretion. A shift in the firing pattern induced by Bay K 8644, from single spiking to plateau AP accompanied with an increase in the frequency of firing in gonadotrophs was sufficient to trigger LH secretion. Although the AP duration in gonadotrophs stimulated with Bay K 8644 was shorter compared with that of plateau-bursting in somatotrophs and lactotrophs, when combined with an increase in the firing frequency it was adequate to elevate $[Ca^{2+}]_i$ and LH secretion to the levels observed in unstimulated somatotrophs and lactotrophs. It should be noted, however, that an increase in spike frequency alone was not sufficient to stimulate LH secretion, as demonstrated by the inability of 5 mM K^+-induced depolarization to stimulate exocytosis in gonadotrophs. In line with this, it has been shown that AP broadening contributes to the frequency-dependent facilitation of $[Ca^{2+}]_i$ signals in pituitary nerve terminals (31).

The ability of somatotrophs and lactotrophs to fire low amplitude plateau-bursting type of APs and gonadotrophs to fire high amplitude single spikes indicates the cell type-specific expression of plasma membrane channels. In general, a similar group of ionic channels are expressed in each cell type, including transient and sustained VGCCs, TTX-sensitive Na^+ channels, transient and delayed rectifying K^+ channels, and multiple Ca^{2+}-sensitive K^+ channel subtypes (3, 8, 18, 27, 38–46). In accordance with the above hypothesis, there were marked differences in the expression levels of some of the ionic channels when analyzed in the same preparation. Specifically, lactotrophs and somatotrophs exhibited low expression levels of TTX-sensitive Na^+ channels and high expression levels of the

large conductance, Ca^{2+}-activated K^+ channel compared with those observed in gonadotrophs. In addition, functional expression of the transient K^+ channel was much higher in lactotrophs and gonadotrophs than in somatotrophs. The expression of the transient VGCCs was also higher in somatotrophs than in lactotrophs and gonadotrophs (27). Within these channels, it appears that BK channel activation in somatotrophs prolongs membrane depolarization, leading to the generation of plateau-bursting activity and facilitated Ca^{2+} entry. Such a paradoxical role of BK channels is determined by their rapid activation by domain Ca^{2+}, which truncates the AP amplitude and thereby limits the participation of delayed rectifying K^+ channels during membrane repolarization. Conversely, pituitary gonadotrophs express relatively few BK channels and fire single spikes with a low capacity to promote Ca^{2+} entry. Elevation in BK channel expression in a gonadotroph model system converted single spiking activity into plateau-bursting activity that had a high capacity to drive Ca^{2+} entry (47).

The cell type-specific AP secretion coupling observed here is consistent with hypothalamic control of pituitary hormone secretion *in vivo*. Initially, it was believed that all anterior pituitary cell types were under dual hypothalamic control by stimulatory and inhibitory factors. This remains true for somatotrophs and lactotrophs, in which the dual hypothalamic control of GH and PRL secretion is well established (reviewed in Refs. 48 and 49). A negative hypothalamic factor controlling gonadotropin secretion, however, has not been identified. Moreover, the data shown here confirm that there is no need for such regulation, because basal LH secretion is very low. The dual control of somatotrophs and lactotrophs is essential for generating the episodic release of GH and PRL (48, 49), whereas the work by Knobil (50) and others (51) has established that the hypothalamic GnRH pulse generator itself accounts for the pulsatile release of LH. Furthermore, episodic LH release is required for normal reproductive functions, and AP secretion coupling in spontaneously active gonadotrophs, like continuous GnRH administration (50), would inhibit the reproductive cycle.

The lack of AP-induced secretion in unstimulated gonadotrophs does not diminish the importance of AP firing in these cells. Although subthreshold for activation of exocytosis, spontaneous VGCI in gonadotrophs maintains the $[Ca^{2+}]_i$ at the optimal level required for interactions between inositol (1,4,5)-trisphosphate and Ca^{2+} in their dual control of inositol (1,4,5)-trisphosphate channel gating (52, 53). Furthermore, GnRH-induced and inositol (1,4,5)-trisphosphate-mediated $[Ca^{2+}]_i$ oscillations in gonadotrophs generate transient V_m hyperpolarizations, upon which bursting firing is observed (23, 24). Although the agonist-induced shift in the pattern of AP firing alone cannot protect against depletion of the intracellular Ca^{2+} stores, it provides a steady-state increase in VGCI during prolonged GnRH stimulation (53). Combined with the redistribution of Ca^{2+} between mitochondria and endoplasmic reticulum (54), such VGCI is sufficient to maintain agonist-induced $[Ca^{2+}]_i$ oscillations and LH release for several hours (19).

In conclusion, our results indicate that spontaneous, extracellular Ca^{2+}-dependent AP firing is a common feature of pituitary somatotrophs, lactotrophs, and gonadotrophs. Such V_m oscillations were sufficient to stimulate GH and PRL but not LH release. This indicates that cell excitability *per se* is not sufficient for an effective AP secretion coupling in excitable endocrine cells as it is in neuronal cells during synaptic transmission. Our results further indicate that the pattern of spontaneous electrical activity encodes the cell type-specific basal hormone secretion. Specifically, somatotrophs and lactotrophs fire plateau-bursting APs with a high capacity to drive Ca^{2+} entry, whereas gonadotrophs fire single spikes with a low capacity to drive Ca^{2+} entry. The cell type-specific AP secretion coupling in pituitary cells described here provides a rationale for the existence of negative hypothalamic control of PRL and GH but not LH secretion.

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